SYNTHESIS OF NASCENT PROTEIN BY RIBOSOMES IN ESCHERICHIA COLI

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Introduction.—Cells and tissues of all kinds of living organisms have been found to contain ribonucleic acid (RNA) and protein both as separate constituents and as complexes in the form of ribonucleoprotein particles. From a variety of experimental data it has been inferred that these particles are probably intimately involved in the processes of protein synthesis.

The term microsome was originally applied to the portion of disrupted animal tissue which sedimented between 20,000 and 100,000 $\times$ g.[1, 2] It was found to contain most of the RNA. Later it was established that microsome preparations consisted of an association of ribonucleoprotein particles with fragments of the endoplasmic reticulum and techniques were evolved for removing the latter to yield particles which contained little else besides RNA and protein (see review by Palade[3]). Similar particles have also been isolated from bacteria[4] and the term ribosome has been suggested for the purified particles which are essentially free from lipid and other extraneous matter.[5]

In bacteria, depending on the cultural conditions, up to 80 per cent of the RNA may exist in varying sized ribonucleoprotein particles. These range in sedimentation constants from about 20S to 100S and some preparations have about 2 amino acid residues per ribonucleotide. The distribution among the various classes found in *Escherichia coli* depends on the cultural conditions and the conditions of cell breakage. Exponentially growing organisms possess ribosomes with sedimentation constants approximately 15–20S, 30S, 50S, 70S, 85S, 100S. The last is usually a minor component or absent except in resting cells and the 70S and/or 85S particles predominate over the smaller sizes.[6, 7] The sedimentation constant of the particle which we refer to as 85S does in fact vary between 70S and 100S depending on the magnesium concentration.[8]

Separation of the various particles into homogeneous fractions presents considerable difficulties if attempted by simple differential centrifugation. We have found, however, that centrifugation of cell juices layered on sucrose solutions in the swinging bucket rotor of the Spinco can give a useful resolution of all classes of ribosomes seen in the analytical ultracentrifuge. In addition the isolated fractions are available for analysis. This technique has been used to observe the incorporation of radioactive tracers into the ribosomes and the soluble protein of *E. coli*.

Methods.—The procedures described below were followed except for minor modifications. A radioactive tracer was added to an exponentially growing culture of *E. coli* and, after an appropriate interval, the organisms were fractionated to determine the distribution of the tracer among the various sizes of ribosomes and in the soluble protein.

E. coli B (ATCC No. 11303) was grown overnight at 37°C in an aerated synthetic medium containing glucose (0.4 mg/ml) as carbon source. The yield of organisms was approximately 120μg dry weight per ml of culture and in the morning more

glucose (0.8 mg/ml) was added. This caused immediate resumption of exponential growth. The organisms were harvested after the culture density had about doubled. The cells were washed twice and suspended in a synthetic medium lacking both glucose and the sulfur source. Glucose (0.8 mg/ml) was added and the cells (suspension density of 250 μg/ml) were incubated with aeration at 37°C. Exponential growth was promptly resumed at the expense of endogenous sulfur reserves.[9]

After 30 minutes the culture was poured into a beaker containing carrier-free $Na_2S^{35}O_4$ at a level of 1 mC per liter of culture. After a few seconds the whole suspension was dumped on to half its weight of crushed, frozen medium. This caused the temperature to drop precipitously and effectively halted metabolic activities instantly.

The organisms were then harvested, washed three times with TSM (tris(hydroxymethyl)aminomethane 0.01 *M*, succinic acid 0.004 *M*, MgAcetate 0.01 *M*, pH 7.6)

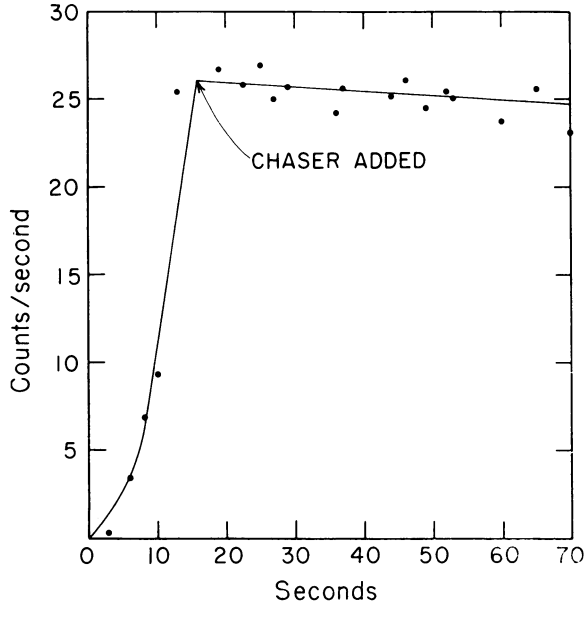

FIG. 1.—Time course of incorporation. $S^{35}O_4^=$ was added to a growing culture of sulfur-depleted *E. coli* at time 0. Samples were withdrawn at indicated times, and squirted into TCA, filtered and counted. At 16 seconds $S^{32}O_4^=$ S^{32} cystine and methionine were added. Note prompt incorporation of the tracer into TCA precipitable material and rapid cessation of incorporation after addition of nonradioactive material.

and resuspended in TSM (7.5 mg dry weight/ml). The cells were broken either by passing through an orifice under high pressure or by the lysozyme-freezing method.[10] Residual cells, cell walls, and membranes were then removed by centrifugation. The resulting cell juice was then ready for sedimentation analysis as follows.

A mixing device[11] was used to deliver 4.5 ml of a solution of sucrose in TSM linearly graded from 20 per cent w/v to 5% w/v into a centrifuge tube. On this was layered 0.5 ml of a solution graded from 4 per cent to 0 per cent sucrose and containing the cell juice in reversed concentration gradient. This system has inherent stability properties which make it valuable for centrifugal separation of components of different sedimentation constants.[12] The tubes were spun in the swinging bucket head rotor (90,000 g) for periods varying from 75 to 200 minutes. Twenty-five fractions were collected from each tube by puncturing the base with a

hypodermic needle and allowing the contents to drip out into small test tubes. The ultraviolet absorption of the fractions was measured. In addition, the radioactivity precipitable by trichloracetic acid (TCA) was determined. All operations except the final collection of the fractions were carried out at 0–4°C.

Results.—As a preliminary to the study of the incorporation into ribosomes, we investigated the kinetics of $S^{35}O_4^=$ fixation into the total cold TCA precipitable fraction of *E. coli* and the rapidity with which this incorporation could be stopped by lowering the temperature or by adding a "chaser" containing non-radioactive $SO_4^=$, cystine and methionine. Samples were removed with a hypodermic syringe as rapidly as possible from a culture after addition of $S^{35}O_4^=$, squirted into TCA, filtered and counted.[13] After an appropriate time the nonradioactive substrate

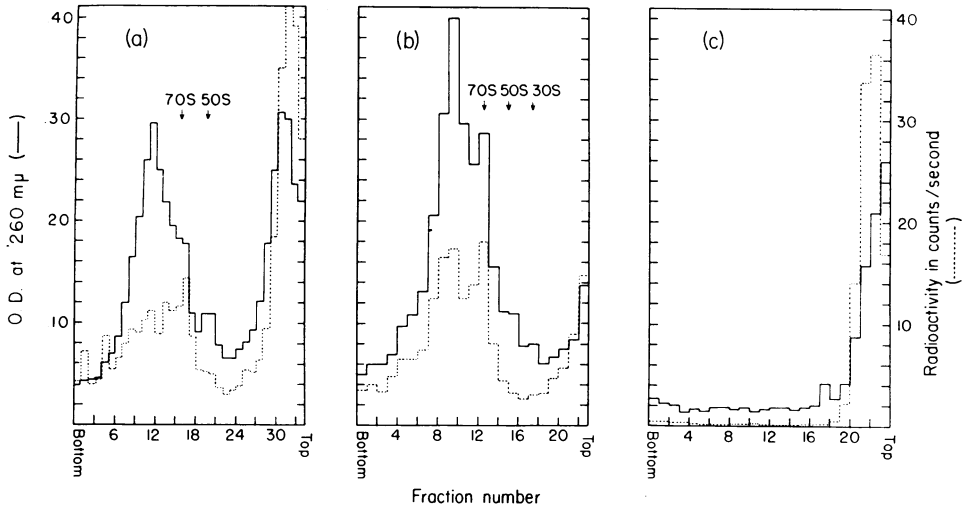

FIG. 2.—Sedimentation analysis of cell juice after 15 seconds incorporation of $S^{35}O_4^=$. $S^{35}O_4^=$ was added to a growing culture of sulfur-depleted *E. coli*. After 15 seconds the culture was chilled, harvested, washed, and broken. The cell walls and membranes were spun out and the juice analyzed in the swinging bucket rotor; 2*a* shows analysis of the total juice; 2*b* analysis of resuspended ribosome pellet; 2*c* analysis of supernatant fluid after removal of ribosomes. The 85S, 70S, 50S, and 30S particles seen in the analytical centrifuge are partially resolved. Note lack of contamination of ribosome region by soluble protein, 2*c*. Centrifugation 75 minutes at 37,000 rpm.

was added and the sampling continued. As shown in Figure 1 a linear rate of incorporation was found after a few seconds and this could be obliterated instantly when the "chaser" was added. These experiments were carried out with cells which had been grown for about 30 minutes without an exogenous source of sulfur. The absence of kinetic delays in the incorporation of S^{35} and in its cessation indicate that under these conditions of sulfur starvation there is little if any pool of precursors to the sulfur amino acids of the cell protein.

In other experiments the culture was poured on ice a few seconds after adding the tracer. Samples taken from the iced suspension during the following 2 hours showed a rate of incorporation less than $^1/_{5000}$ of the rate at 37°. Furthermore, none of the S^{35} which had been previously incorporated into TCA precipitable material was removed by exchange in the presence of chaser at the low temperature.

Accordingly, changes in the content of TCA-precipitable S^{35} occurred during the period when the cells were incubated at 37° and not during the period required for harvesting, washing, and fractionating the cells, all of which were carried out between 0–4°C.

Experiments of a few minutes' duration had consistently failed to show a higher specific radioactivity in the ribosomes than in the soluble protein. We, therefore, investigated the distribution of S^{35} after very short times of incorporation. Organisms were incubated for 10 seconds with $S^{35}O_4^=$ and their components were separated by sedimentation analysis. The distribution of TCA-precipitable radioactivity and 260 mμ absorbing material is shown in Figure 2a. Most of the S^{35} is seen to be associated with the ribosomes of the 70–85S class and with the soluble protein which did not sediment under the conditions used. When the ribosomes were pelleted from such a juice and the resuspended pellet similarly analyzed, most of the soluble protein fraction was eliminated but the 70S particles still carried their

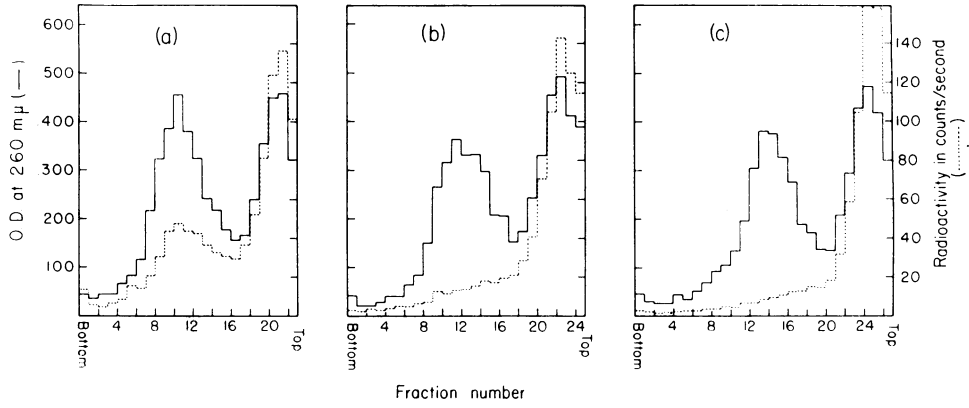

FIG. 3.—Sedimentation analysis of cell juice. (*A*) Cells incubated 15 seconds with $S^{35}O_4^=$. (*B*) Cells incubated 15 seconds with $S^{35}O_4^=$ followed by 15 seconds incubation with S^{32} "chaser." (*C*) Fifteen seconds incubation with $S^{35}O_4^=$ followed by 120 seconds with S^{32} "chaser." Note transfer of radioactivity from 70–85S region to nonsedimenting region. Centrifugation 75 minutes at 37,000 rpm.

original radioactivity (Fig. 2b). This indicates that the S^{35} was relatively firmly bound to the ribosomes (see also below). When the supernatant fluid remaining after the ribosomes had been pelleted was analyzed, very little of the radioactivity moved down the centrifuge tube (Fig. 2c).

In a variety of experiments of short duration (5–20 seconds) the major part of the label associated with ribosomes was found in the 70–85S fraction and the specific radioactivity of these particles was always substantially greater than that of the 30 and 50S ribosomes. When incubations lasting 20, 45, and 120 seconds were used and the resulting cell juices analyzed, it was found that the specific radioactivity of the 70S particles had risen rapidly by 20 seconds and thereafter increased only a little up to 120 seconds. The specific radioactivity of the 50S ribosomes rose more slowly but surpassed that of the larger particles between 45 and 120 seconds. The soluble protein also became labeled rapidly but because of the much greater amount had lower specific radioactivity at the earlier time.

To demonstrate that one component of the cell acts as precursor to another it is necessary to show not only that the radioactivity of the suspected precursor rises rapidly when a tracer substrate is added but also that it is transferred to the product with equal rapidity when the radioactive substrate is replaced by a nonradioactive one. Cells were therefore incubated with the radioactive tracer for 10–15 seconds and then a "chaser" of nonradioactive amino acids was added. After a short period all incorporation was terminated by pouring the culture on ice. Figure 3 shows that most of the radioactivity which was found in the ribosome fraction at the end of 15 seconds was subsequently transferred to the soluble protein during the 15 seconds of incubation with the "chaser." The removal was only slightly greater in 120 seconds. Even a 5 second "chase" was quite effective. Thus there is a protein component which is transiently associated with the ribosomes and has all

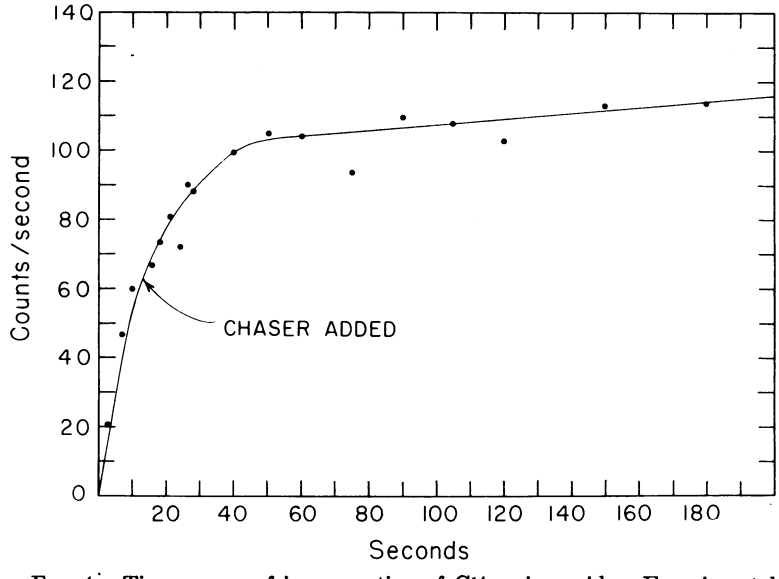

FIG. 4.—Time course of incorporation of C^{14} amino acids. Experimental conditions similar to those of Fig. 1 except C^{14} labeled chlorella protein hydrolysate was used as tracer. Note that incorporation continues after addition of C^{12} amino acids.

the characteristics which would be expected in a compulsory precursor of the soluble proteins. It appears that this nascent protein is a polypeptide strand which is formed on the ribosome and is subsequently released as soluble protein. Owing to the short time periods involved it has not been possible to plot detailed time courses of these processes but three features have been established. (1) The radioactivity of the 70–85S ribosomes built up to a saturation level in 5 seconds or less and died away equally rapidly when the tracer was diluted out. (2) The saturation level was equivalent to the quantity of soluble protein synthesized in three seconds. (3) The decrease in the radioactivity of the ribosomes during the chase was roughly equal to the concomitant increase of radioactivity in the soluble protein.

To check whether the results obtained with S^{35} incorporation were typical of other

amino acids, similar experiments were carried out to observe the incorporation of a mixture of amino acids.† Figure 4 shows that the incorporation of C^{14} from the mixture of C^{14} amino acids (obtained by hydrolyzing *Chlorella* protein) began promptly but could not be terminated rapidly by adding a large excess of C^{12} amino acids. Exchange of exogenous amino acids with the amino acids of the pool is not very rapid and C^{14} continued to enter the protein fraction for roughly 20 seconds after the "chaser" was added. Consequently a longer period was needed to show the transfer of the particle-bound nascent protein to the soluble fraction. The results, however, were quite similar to those obtained with S^{35}. After 5 and 12 seconds a large fraction of the C^{14} incorporated into the TCA-precipitable fraction was associated with the 70–85S particles as shown by the sedimentation analysis. During the subsequent 120 seconds after the addition of C^{12} amino acids the C^{14} was transferred

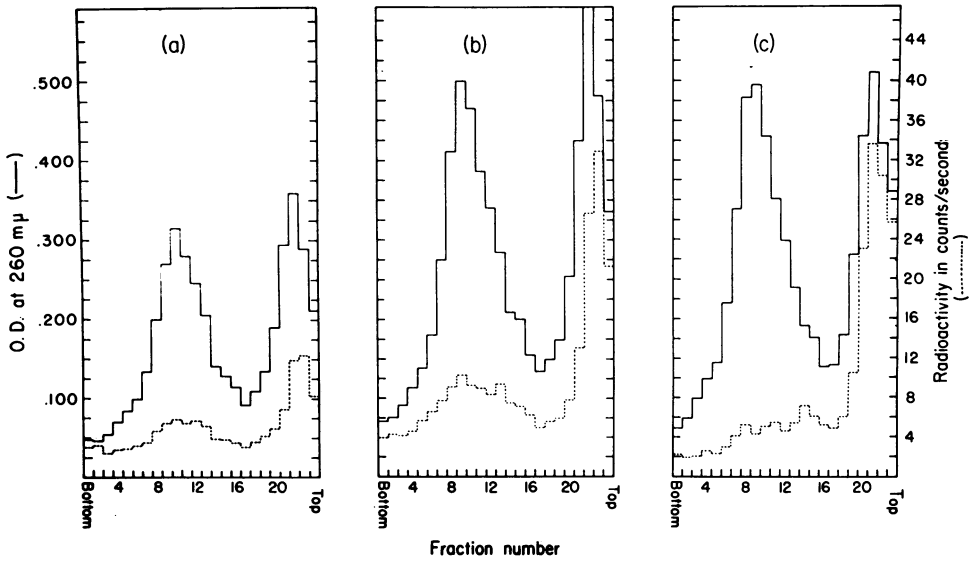

FIG. 5.—Sedimentation analysis of cell juice. (*A*) Cells incubated 5 seconds with C^{14} amino acids. (*B*) Twelve seconds incubation. (*C*) Twelve seconds incubation with C^{14} amino acids followed by 120 seconds incubation with C^{12} amino acids. Note decrease in radioactivity of 70–85S region and increase of radioactivity in nonsedimentable region. Centrifugation 75 minutes at 37,000 rpm.

to the soluble fraction (Fig. 5). As a result of the longer times required to dilute out the free amino acid pools there was more opportunity for incorporation into the structural proteins of the ribosomes and the transfer was not quite so complete as could be observed when using the S^{35} tracer. Neither was it possible to demonstrate the rapidity of the transfer from the particle to the soluble fraction. Nevertheless the results show that the behavior of the S^{35} amino acids is consistent with that of the other amino acids and can be used with confidence to study the processes of protein synthesis.

In the experiments described above we have taken TCA-precipitability as an indication that the labeled amino acids were in peptide linkages. There exist, however, complexes of soluble RNA or lipids with amino acids which are precipitated by cold TCA. A number of tests were therefore carried out to establish the nature

of the labeled material which is transiently associated with the ribosomes. (1) It remained precipitable by TCA after solution in 1 *N* NaOH. (2) It was not extractable by hot alcohol after cold TCA precipitation. (3) It was not exchangeable by incubation with an excess of nonradioactive amino acids. (4) It yielded a large variety of compounds (peptides) having different electrophoretic mobilities after partial hydrolysis by chymotrypsin or 12 *N* HCl. These tests indicate that the bulk of the newly incorporated amino acids which were found associated with the ribosomes have the behavior to be expected of amino acids bound in the peptide linkage.

The association of the nascent protein with the ribosomes, although transient in the growing cell, is quite stable in the disrupted cell juice. Preparations have shown the same specific activity in the ribosome fraction after 5 days at 4°C.

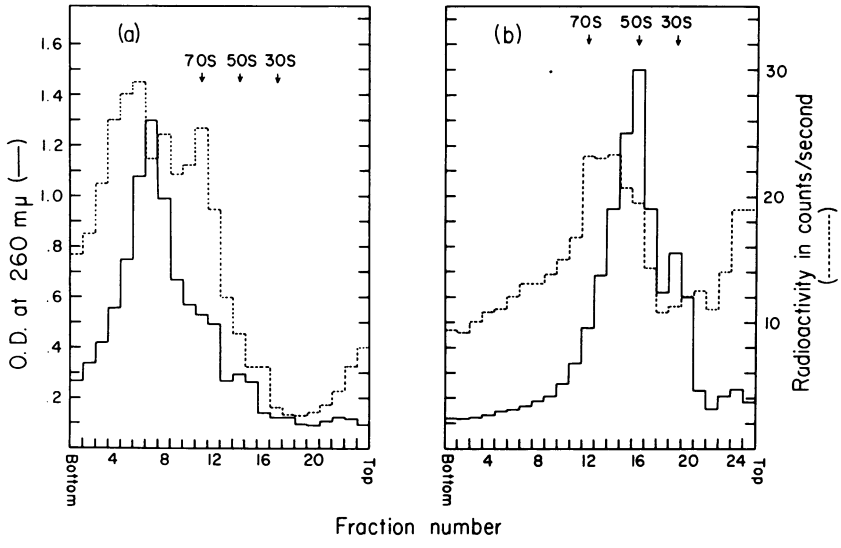

FIG. 6.—Sedimentation analysis of ribosomes from cells exposed for 10 seconds to $S^{35}O_4^{=}$. (*A*) Ribosome pellet resuspended with buffer containing Mg at 10^{-2} M to give mostly 70–85S ribosomes. (*B*) Pellet resuspended with Mg at 10^{-4} M to give mostly 50S and 30S ribosomes.

The ribosomes may be centrifuged down from the cell juice and the pellet shows the same specific radioactivity in the 70S region upon subsequent sedimentation analysis. We have not yet found conditions which cause the release of the nascent protein from the ribosomes. Incubation (15 minutes at 37°) with adenosine triphosphate (ATP) or with amino acids in the presence of cell juice did not release it. Chromatography with diethylaminoethyl (DEAE) cellulose caused disintegration[14] of the ribosomes yielding nucleoprotein which was eluted from the column but leaving about one-half of the ribosome protein and 95 per cent of nascent protein firmly bound. On splitting the ribosomes by decreasing the magnesium concentration of the medium,[15] the nascent protein was found partly in the soluble proteins but mainly in the 50 and 30S particles which comprised the bulk of the nucleoprotein and in a few remaining 70S ribosomes (Fig. 6).

On the other hand, the soluble protein which was labeled with S^{35} as early as 7

seconds after addition of the tracer could not be distinguished from the bulk of the unlabeled soluble protein by chromatography on DEAE. There is no indication from column chromatography that any appreciable time is required (in growing cells) for conversion from nascent protein associated with the ribosomes to the final form of the polypeptide chain.

A less detailed study was made of the incorporation of S^{35} in the fraction of broken cells which sedimented rapidly. This contained fragments of cell walls and membranes as well as some intact cells. Under steady state conditions of labeling roughly 25 per cent of the total S^{35} was in this fraction whereas organisms which had been incubated with tracer for only 10–15 seconds showed about 30 per cent. This might be taken to indicate the presence of a protein precursor. However, sedimentation analysis indicated that half of this material sedimented very rapidly (>1000S) as might be expected for cell walls and unbroken cells. A large part of the remainder is accounted for by contamination with ribosomes and soluble protein leaving only a minor component which has the sedimentation properties to be expected of small fragments of cell membrane. The specific radioactivity of this fraction was similar to that of the soluble protein. Furthermore, its radioactivity did not diminish after the addition of a "chaser." Thus, there is no evidence that this fraction contains a major protein precursor. There is, however, a slight indication that ribosomes associated with cell membranes may be more active in protein synthesis. When cells were lysed by treatment with lysozyme followed by freezing and thawing about half of the ribosomes were released and most of the remainder were detached from the residual membranous material by passing it through the pressure cell. It was found that the first fraction of ribosomes had only about half the specific radioactivity of the second. Possibly some of the particles exist free in the cell juice whereas others are more or less firmly bound to membranes and are more directly involved in protein synthesis.

Discussion.—Various lines of evidence suggest that the microsome system in animal cells may be the predominant site of protein synthesis. For instance, amino acid incorporation studies *in vivo* show that this cell fraction becomes labeled most rapidly and only subsequently does the soluble protein fraction become radioactive.[16,17] Similar results have been obtained with pea seedling preparations.[18] Support for these ideas has come from *in vitro* experiments in which suitably supplemented microsome preparations have been shown to incorporate amino acids into peptide linkage.[19] However, the incorporation was relatively small and boiled preparations were reported to be substantially more active.[20]

In contradistinction, studies with bacterial cell preparations had not hitherto indicated that ribosomes were an important site of protein synthesis. This is surprising in view of the fact that bacteria have a considerably higher content of ribosomes than do animal cells. Some recent reports, in fact, propose that the bacterial cell membrane is a relatively more important structure in such synthesis.[21–23]

In animal tissues, cellular proliferation is small compared with synthesis of soluble protein so there is little difficulty in distinguishing between synthesis of and by ribosomes. In certain cells there is no synthesis of ribonucleoprotein particles so that all incorporation of amino acids can be attributed to synthesis of soluble protein by the ribosomes.[24] In contrast, in growing cultures of bacteria the ribosome content will double in a mean generation time as will the amount of soluble protein.

E. coli in the exponential phase of growth in a synthetic medium has a mean generation time of about 50 minutes and contains approximately 40 per cent soluble protein, 10 per cent ribosomal protein, and 10 per cent cell wall protein, all expressed on a dry weight basis. In a single cell there are roughly 6000 of the 70–85S ribosomes and 6×10^6 arbitrary units of 10,000 MW in the soluble protein. As the growth rate is 0.02%/second, 1.2 ribosomes and 1200 protein units will be synthesized per second. Assuming that the ribosomes are the site of protein synthesis there may be as many as 6,000 sites operating. If this were so, the average time required for the completion of a protein unit would be five seconds. If these nascent protein molecules leave the ribosomes as soon as they are completed, then only those protein molecules synthesized during the previous five seconds will be found adhering to the ribosomes.

At the same time amino acids are flowing in to form the structural proteins of the ribosomes at one-quarter the rate that amino acids are required for synthesis of soluble protein. Hence after twenty seconds the total quantity of new ribosomal protein will equal the quantity of adhering nascent protein. Earlier experiments[14] failed to detect the presence of the nascent protein because periods as long as four minutes were allowed for incorporation. At the end of four minutes the nascent protein would be only 10 per cent of the newly formed ribosome protein.

Fortunately, techniques are available to recognize these two types of synthesis. A 5–15 second "chase" will displace the nascent protein but does not affect the newly formed ribosome protein which is a permanent end product. In other studies carried out in this laboratory[25, 26] it was found that longer incubations (1–30 minutes) with $S^{35}O_4^=$ followed by a short "chase" gave a pattern of labeling which implied that the smaller ribosomes were made first and were themselves precursors to the larger, 30 and 50S particles combining to yield 70 and 85S particles. Accordingly, the two types of synthesis are partially separated according to particle size, the larger particles carrying most if not all of the nascent protein whereas amino acids newly incorporated into the structure of the ribosomes appear at first in the smaller particles. Furthermore, the use of $S^{35}O_4^=$ as a tracer emphasizes the nascent soluble protein as the sulfur amino acid content of the ribosomal protein is one-half to one-third lower than that of the soluble protein. As a result, the radioactivity appearing in the 70–85S particles after incubation with $S^{35}O_4^=$ for 15 seconds or less is chiefly due to nascent protein and there is little contamination from protein of newly formed ribosomes.

The results reported here indicate that a time scale of 5 seconds for the completion of protein units is approximately correct. Free amino acid pools might introduce some slight kinetic delays which would increase the apparent time but there is no doubt that the nascent protein has a transient existence of less than 5–10 seconds in the growing cell.

It is not possible from these experiments to determine the size of the polypeptide strands which make up the nascent protein as this calculation requires a knowledge of the fraction of the ribosomes that is active. The 30 and 50S ribosomes are certainly not the major loci of the nascent protein but they are only minor components and it cannot be excluded that they carry a correspondingly small part of the nascent protein. Their role in forming nascent protein is obscured by the rapid labeling of their structural protein.

It is possible that both the 70 and 85S particles synthesize nascent protein but the former consistently show an initial higher specific radioactivity. Hence, it seems more likely that the nascent protein is formed on the 70S particles and is carried on to the 85S by a rapid interchange between these forms. Finally, it is also possible that only the 70S particles associated with the cell membrane are active. If we assume, however, that the 70S particles are all equally active and that the other particles are inert, and if we take 5 seconds as the time for formation of a polypeptide strand, then the product must have a molecular weight of roughly 20,000.

At first sight it might appear that a growing culture of bacteria is a system so complicated as to be less useful in a study of the problems of protein synthesis than are more simplified systems. It has become apparent, however, that by choosing appropriate time scales one can emphasize one or another aspect of the total cell synthesis. When this is done it is possible to show both the rapid build-up and decay of radioactivity in the nascent protein attached to the ribosomes and to show its transfer to the soluble protein. Furthermore, it is possible to show that this process occurs in the appropriate quantity to account for most if not all of the synthesis of the soluble protein. Finally, it is possible to study separately the synthesis of protein by ribosomes and the synthesis of the ribosomes themselves even though they are intimately coupled in the intact growing cell.

Summary.—The ribosomes of *Escherichia coli* can be separated into different sedimentation classes by centrifugation of cell juices through solutions of sucrose.

Radioactivity from $S^{35}O_4^=$ and C^{14}-amino acids rapidly appears in protein bound to the 70S and 85S ribosomes. These become saturated within a few seconds and equally rapidly shed this nascent material as soluble protein which cannot be distinguished from the main bulk of soluble protein.

The rate of formation of this nascent protein on the larger ribosomes is adequate to account for the total cell protein synthesis.

This process is distinct from the concomitant synthesis of the proteins which constitute the permanent structure of the ribosomes.

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† These experiments were carried out with Dr. R. Hendler who used a portion of the cultures to measure the kinetic behavior of lipid-bound amino acids.

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*THE LOWER RANGE OF THE COCHLEAR POTENTIALS**

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Many experiments have been concerned with the functional relation between sound stimuli and the magnitude of the cochlear potentials. Early studies indicated that this function is linear, or nearly so, over a wide range, but departs from linearity when the applied sounds reach extreme intensities. Wever and Bray in 1936, in observations on the guinea pig, obtained curves whose linear range extended from 1 to 150 microvolts at 1,000 cycles per second and from 1 to 100 microvolts at 4,000 cycles, a range of 40 db or more. With the equipment used at that time it was not possible to obtain reliable measurements below 1 microvolt, yet it was pointed out that this value did not represent a lower limit for the response, and doubt was expressed whether any limit exists.

Further studies with improved equipment, and especially with the wave analyzer as a selective voltmeter, extended these measurements a decade lower, to the region of 0.1 microvolts. In this region the function was found to continue its linear form for all tones and in all animal species, including mammals, birds, reptiles, and amphibians. (See, for example, recent studies on cats by Wever, Vernon, Rahm, and Strother, on turtles by Wever and Vernon, and on frogs by Strother.)

Despite this evidence there have been some who have spoken of a "threshold" for the cochlear potentials. Careful consideration shows that in these instances the investigators were misled by the limitations of their measuring equipment, and their "thresholds" merely represented the lowest values that could be read under the conditions of the experiment. The limitations were imposed by background